Modulation of the immune system during post-partum uterine inflammation.

Running Title: Transcriptional regulation of uterine inflammation

Key words: DNA methylation, Gene expression, Dairy cow, Endometritis, Inflammation.

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Abstract

Post-partum uterine inflammation (endometritis) in the dairy cow is associated with lower fertility at both the time of infection and after the inflammation has resolved. We hypothesized that aberrant DNA methylation may be involved in the sub-fertility associated with uterine inflammation. The objective of this study was to characterize genome-wide DNA methylation and gene expression in the endometrium of dairy cows with sub-clinical endometritis (SCE). Endometrial tissues were obtained at 29 days post-partum (n=12) and microarrays were used to characterize transcription and DNA methylation. Analyses revealed 1,856 probes differentially expressed in animals with SCE (n=6), compared with controls (CON, n=6, P<0.05, Storey Multiple testing correction) and 2,976 probes with significant correlation between gene expression and bacteriology score. No significant associations among DNA methylation and gene expression were detected. Analysis of

transcription data using GeneGo Metacore and Gene Set Enrichment Analysis identified several pathways and processes enriched in SCE cows, with the majority related to the immune response. Further, the top ontology terms enriched in genes that had expression data correlated to bacteriology score were: Defence response, inflammatory response, and innate immune response. Gene expression profiles in cows with subclinical endometritis in this study indicate that the immune response is activated, potentially resulting in a local pro-inflammatory environment in the uterus. If this period of inflammation is prolonged it could result in tissue damage or failure to complete involution of the uterus, which may create a sub-optimal environment for future pregnancy.

Introduction

Uterine infection following parturition is a common cause of sub-fertility in dairy cows (11, 40, 58). Infection resulting from microorganisms entering the uterus can result in an inflammatory state that, if left unresolved, can negatively affect subsequent reproductive performance. Following parturition, the uterus undergoes a period of extensive remodelling or involution. Involution involves physical shrinkage of the uterus, followed by necrosis and sloughing of the caruncules, and lastly regeneration of the endometrium. During the early stages of involution, the uterus has a disrupted epithelial surface, coupled with fluid and tissue debris that support bacterial growth (32). Structural and functional effects of bacterial infection on the endometrium likely contribute to reduced fertility. Other causes of post-partum inflammation include those resulting from systemic metabolic perturbation during the transition period. During the early post-partum period dairy cows undergo a period of negative energy balance (51). There is some evidence that this negative energy balance is associated with reduced immune function and increased inflammation in the uterus (23, 68, 69).

Failure to resolve postpartum uterine inflammation, an endometritis, can delay uterine involution and reduce reproductive function, including: suppression of hypothalamic gonadotropic secretion, follicular growth and function, and ovulation, as well as reduced conception and pregnancy rates (7, 33, 34). Furthermore, even sub-clinical endometritis, defined as inflammation of the uterus without clinical signs of endometritis, is negatively associated with subsequent reproductive performance (6, 21, 27).

Many transcriptional changes occur in the uterus during involution and these are likely regulated by epigenetic mechanisms (67). Epigenetic mechanisms such as DNA methylation regulate gene expression and, subsequently, phenotype without changing the underlying DNA sequence. There is compelling evidence that variation in the immune response in cattle is epigenetically regulated (22, 54). Further, we have previously reported an association between DNA methylation and gene expression in the endometrium during early pregnancy (65). Therefore it was hypothesized that prolonged post-partum uterine inflammation may be the result of maladaptive DNA methylation of inflammatory mediators. If this epigenetic landscape persists, it could explain the sub-fertility associated with post-partum inflammation.

To understand the molecular mechanisms regulating post-partum inflammation, we characterized gene expression in the endometrium of animals with or without a post-partum uterine inflammation.. Furthermore, it was hypothesized that aberrant DNA methylation may be involved in the sub-fertility associated with post-partum uterine inflammation. To test this hypothesis, genome wide gene expression and DNA methylation were characterized in the uterus of dairy cows with and without sub-clinical endometritis at 29 days post-partum.

Methods

Animal trial

All procedures were undertaken with the approval of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). The experiment was undertaken at Scott Farm (37°45.952'S, 175°21.835'E; DairyNZ Ltd, Hamilton, New Zealand) using 109 cows. All cows grazed pasture and were managed in an intensive rotational grazing manner (47). Cows in both groups were matched for calving date, breed and age. Those with calving difficulties or retained placenta, or that had been treated with antibiotics during the first 30 days post-partum, were excluded from the study.

Uterine sampling and polymononuclear cell counts

Uterine sampling was undertaken between 22 and 25 days post-partum. Prior to uterine samples being collected, the vulva was cleaned. Collection of uterine samples for evaluation

of polymorphonuclear (PMN) cells and uterine bacteriology was undertaken as described previously (47) using pap endocervical sample brush (cyto-brush, Ebos Group Ltd, Christchurch, New Zealand). The sterile cyto-brush was mounted on to a sterile stillette (length: 500 mm; internal dia. 5.0 mm; external dia. 7.0 mm). The stillette and cyto-brush were protected by a cannula, which in turn, was protected by a plastic sleeve. The cannula was inserted into the uterus via the cervix. Once in the lumen of the uterus the cyto-brush was passed through the plastic sleeve and rotated a quarter turn while in contact with the endometrium. The cyto-brush was retracted into the cannula tip before withdrawal from the uterus, cervix and vagina. The brush was smeared onto a slide that was air dried and stained with Diff-Quik® Stain (Dade Behring, Newark, DE, USA.) within 1 h of sample collection. Cytological examination was completed within 24 to 48 h. More than 100 nucleated cells were assessed and the percentage that was PMN and macrophages were recorded.

Bacteriology

Bacteriology was performed as previously described (39). Briefly, a cytobrush (Pap endocervical sample brush, Ebos Group Ltd, Christchurch, New Zealand) was used to collect uterine samples for bacteriology analyses at the same time as uterine cytology was performed. The cytobrush sample was then placed in Stuart Transport media (Copan Ames without charcoal, Oxoid, Basingstoke, UK). The cytobrush was removed from the transport media within an anaerobic chamber (80% N_2, 10% CO_2, and 10% H_2 atmosphere; Coy Laboratory Products, Grass Lake, MI, USA) and streaked across two 0.1% esculin, 5% sheep blood agar plates (Fort Richard, Auckland, New Zealand). One plate was then removed and incubated aerobically. The end of the cytobrush was then snapped off and immersed in a fastidious anaerobic broth (Fort Richard, Auckland, New Zealand). Bacterial growth was assessed after 2 and 5 d of incubation at 37 °C in the aerobic and anaerobic environments, respectively. Colony morphology, size, hemolysis patterns, pigmentation, opacity, and consistency were assessed, and each colony type was Gram-stained and cell morphology examined. Further biochemical tests were undertaken as required, using established techniques (35, 39). Gram positive cocci were assessed using catalase and esculin tests. Gram negative rods were evaluated with catalase, lactose, OUMI, and triple iron sugar tests.

The Gram positive coccibacillus were evaluated using catalase, maltose, mannitol, raffinose, and gelatin tests. Each isolate was subcultured to ensure a pure growth and a number of colonies were placed into 2 mL brain heart infusion broth supplemented with 20% glycerol and preserved at −20 °C.

The DNA was extracted from the fastidious anaerobic broth that had bacterial growth, using the procedure for Gram negative bacteria, as per the manufacturer's instruction (Wizard Genomic DNA purification kit, Promega, Madison, WI, USA). This DNA was then analyzed by PCR using primers designed to detect *Prevotella* spp. (71) and *Fusobacterium* spp. (45).

A bacterial score was calculated based on growth in each of the 9 culture conditions, with a maximum score of 9 for cows that had growth in every type of culture (39).

Blood sampling

Blood was collected from the cows on day of euthanasia using coccygeal venipuncture into vacutainer tubes and immediately placed in iced water. The blood samples were centrifuged within 30 min (12 min at 1,500 x g) and the aspirated plasma fractions were stored at -20°C.

Progesterone assay

Progesterone was measured in plasma using a commercial kit (Progesterone Coat-A-Count; Siemens, Los Angeles, CA). The average intraassay coefficients of variation for the high and low controls were 5.91% and 3.14%, respectively, and the minimum detectable level was 0.037 ng/mL.

Selected CON and SCE cows

The criteria for cow selection were based on the uterine swab samples. CON had no PMN or macrophages whereas SCE had > 18 % PMN cells (17) with an absence of macrophages. Cows were allocated to CON and SCE pairs balanced for calving date, breed, and age. A total of 12 cows (CON n=6; SCE n=6) between 27 and 31 days post-partum were sampled in the study. Cows were slaughtered at a commercial abattoir, and blood samples and tissues were collected (42).

Milk yield, composition and body condition

Milk yield (kg/d) was measured twice daily using the DeLaval Harmony and ALPRO® milking system. Milk composition was determined weekly using infrared analysis (FT120m, Foss Electric, Hillerød, Denmark) and somatic cell number was determined using an electronic flurometric cell-counting technique (Fossomatic, Foss Electric, Hillerød, Denmark) by LIC Ltd (Hamilton, NZ). Individual body condition and body weight were measured at 3 time points post-calving (1, 2 and 3 weeks). Body condition was measured on the 10 point scale developed by Roche et al 2004, where 1 is emaciated and 10 obese (50).

Plasma metabolites

Plasma samples were analysed for blood metabolites using colorimetric techniques at 37°C with a Hitachi Modular P800 analyzer (Roche Diagnostics, Indianapolis, IN) as described previously (52). Briefly, the concentration of non-esterified fatty acids (NEFA, WAKO, Osaka, Japan), Magnesium (xlidyl blue reaction), albumin (bromocresol green reaction), total protein (Biuret reaction method) and globulin (calculated by subtracting the albumin value from the total protein value) were measured. Glutamate dehydrogenase (catalysing activity of NADH-dependent conversion of α-ketoglutarate to glutamate) and aspartate aminotransferase (catalysing activity of transamination of L-aspartate to oxaloacetate) were also measured. The inter-assay and intra-assay CV for all assays were ≤11% and ≤2%, respectively.

Tissue sampling

Cows were slaughtered and within 40 minutes of exsanguination, their reproductive tracts were recovered and stored on dry ice for inspection and dissection as follows. Excessive connective tissues were removed from the reproductive tract and the uterine horns were separated. Each uterine horn was dissected separately, and any abnormalities noted. Caruncular and intercaruncular endometrial tissue was sampled from the middle section of each uterine horn. Samples (between 0.5 to 1.0 g) were immediately transferred into cryo-tubes and frozen in liquid nitrogen before storage at -80°C within 70 min of exsanguination. A complete cross-section was placed in neutral buffered formalin (4% w/v; Ajax Finechem Pty Ltd, Auckland, New Zealand) and then embedded in paraffin for histological analysis.

Histology

Sections of caruncular endometrium were examined to determine the degree of inflammation at the time of tissue sampling. Paraffin-embedded uterine tissues were sectioned at 4-8 µm thickness, deparaffinised with xylol and stained with Haematoxylin and Eosin (H&E). The slides were incubated in double strength Gills Haemotoxylin for 3 minutes and washed with running tap water. They were then dipped in acid alcohol (1%) twice, rinsed in running tap water, and incubated in Scotts tap water substitute for 3 seconds followed by 5 minutes in running tap water. Slides were stained in aqueous Eosin (2%) for 3 minutes, rinsed in tap water and drained. The samples were then dehydrated through 95% and 100% ethanol three times, dipped in xylol three times, and mounted with resin and a coverslip. Slides were left to harden overnight and sent to Gribbles Veterinary (Auckland, New Zealand) for inflammatory scoring.

The tissues were assessed using the criteria described by Bonnet et al (9, 10) with some modifications. Epithelial and stratum compactum inflammatory cells were measured at number per number of 400X fields assessed; epithelium was only reviewed in areas where epithelium was at least partly or closely attached. Up to ten representative (every other contiguous) 400X fields of stratum compactum were reviewed per section.

RNA extraction

Endometrial tissues were homogenized in Qiagen buffer RLT (Qiagen GmbH, Qiagen Strasse 1, 40724 Hilden, Germany) using a TissueLyser II (Qiagen). Approximately 30 mg of tissue was homogenized for 2 minutes with three ball bearings of 1/8 inch diameter (Farrell Bearings, Hamilton, NZ) in a tube containing RLT lysis buffer (Qiagen). Total RNA was extracted using a Qiagen RNeasy kit (Qiagen). RNA quantity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). RNA integrity was assessed with a RNA 6000 Nano LabChip kit using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All samples had an RNA integrity number above 7.

Gene expression microarray

RNA (150 ng) were amplified using the Agilent Low Input Quick Amp Labeling Kit, two color (Agilent, G2519F) to generate fluorescent complimentary RNA (cRNA) according to the

manufacturer's instructions. cRNA quantity was measured by spectrophotometry using a ND-1000 (Nanodrop Technologies, Wilmington, DE). Cy3 and Cy5 labeled cRNA (825 ng) were added to Agilent 4x44k 60-mer oligonucleotide microarrays (G2519F 023647) and hybridized overnight at 65°C (17 hours). Slides were then washed with Agilent Gene Expression Wash Buffers 1 and 2 according to manufacturer's instructions, inserted into a slide cover with ozone barrier, and scanned on an Agilent C scanner at 5µm.

Hybridization design

A total of 24 microarrays were used in this study, one for each tissue type among the 12 animals. A reference sample was prepared from equal amounts of RNA from the 24 endometrial samples analyzed in this study. This pooled sample was used as a 'reference' within each array hybridization. The reference sample was labeled with the Cy3 NHS ester dye, while each individual sample was labeled with the Cy5 NHS ester dye.

Data analysis and statistics

Agilent feature extraction software version 10.7.3.1 (Agilent Technologies, Palo Alto, CA, USA) was used to analyze the scanned Agilent microarray. The 24 scanned microarray image files were uploaded to the feature extraction software. Using a design file (023647), the feature extraction software locates features and converts the extracted data from each feature into a quantitative $\log_2$ratio. This software removes pixel outliers, performs statistical tests on the non-outlier pixels, subtracts background from features and flags any outlier features. The software was then used to perform LOWESS (locally weighted linear regression analysis) dye normalization and to calculate a p-value for each feature.

Data analysis was performed on Genespring GX 12.5 (Agilent Technologies, Palo Alto, CA, USA). Microarray data were imported into Genespring using Agilents Data Import Wizard for Agilent Two Color Expression Data. Filters applied to the data to improve the quality of the normalized dataset included: firstly, filtering 'on flags' to ensure any probes that were not deemed 'Detected' (according to feature extraction spot quality guidelines) in at least 100% of at least one experimental group were omitted from analysis; secondly, probes were filtered on raw intensity units using the default setting (lower cutoff: 20^{th} percentile and upper cutoff: 100^{th} percentile). Probes that were not within this range in 100% in at least one experimental sample group were excluded. After quality control filtering 26,026 probes remained and were used for analysis. Comparisons among experimental groups (CON and

SCE) were undertaken using ANOVA, with significance thresholds set at $P<0.05$; multiple testing correction (Storey) was also applied. Gene expression data were also analyzed for association of gene expression and bacteriology score. A linear regression analysis was used to test for any associations, including Benjamini-Hochberg multiple testing correction. The microarray data were uploaded to NCBI Gene expression omnibus (GSE58794). For probes that were not annotated, full length transcripts were identified, where possible, by querying microarray probe sequences against the bovine genome (Btau5.1) using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Gene function and pathway identification

Functional analysis was performed using MetaCore (GeneGo Inc) GSEA (Gene set enrichment analysis and DAVID (Database for Annotation, Visualisation, and Integrated Discovery) (60). Gene lists containing differentially expressed genes for each comparison tested in the ANOVA were used for analyses with MetaCore to identify significantly enriched pathway maps. All entities in the microarray were used for GSEA analysis. GSEA ranks genes based on the correlation between their expression and the class distinction. Given an *a priori* defined set of genes, GSEA determines whether the members of the gene set are randomly distributed throughout the ranked gene list or are primarily found at the top or bottom of the list. An enrichment score (ES) is calculated that reflects the degree to which the gene set is overrepresented. The statistical significance (nominal P-value) of the ES was estimated using a gene set-based permutation test. The estimated significance is adjusted for multiple hypothesis testing by normalising the enrichment score (NES) and then calculating the false discovery rate (FDR) corresponding to each NES. The GSEA user manual recommends an FDR cut-off of 25% for significant enrichment of a particular gene set using the gene set permutation based method. In this study, an FDR cut off value of 0.05 was used. DAVID was used to identify significant GO (gene ontology) terms and KEGG (Kyoto encyclopaedia of genes and genomes) pathways in the list of genes that had gene expression associated with bacteriology score. The EASE test and a multiple testing correction (Bejamini) were used to identify significantly enriched functions. All genes on the microarray were used as the background for this analysis.

cDNA synthesis

Two µg of each endometrial RNA sample (final volume = 40 µL) were used for cDNA synthesis using the Invitrogen Superscript III Supermix kit (Invitrogen Corporation, Carlsbad, CA). Total RNA was transcribed according to the manufacturer's instructions using 27µM of random pentadecamers. Briefly, RNA and random primers were mixed and denatured at 65°C for 5 minutes, followed by 1 minute on ice. Annealing buffer and Superscript/RNase was added to samples and incubated for 10 minutes at 25°C (primer annealing), followed by 50 minutes at 50°C and 5 minutes at 85°C to inactivate the enzyme. Reverse transcription controls were performed, whereby the above process was completed without the addition of superscript enzyme.

Quantitative Real Time PCR

Real time PCR using the Roche Lightcycler 480 was performed using the Roche real time PCR master mix (Lightcycler 480 Probes Master) in combination with Roche Universal Probe Library (UPL) assays (Roche diagnostics, Indianapolis, IN, USA). Assays were designed using Roche UPL design software. All assays were designed to span an intron-exon boundary.

The PCR reaction volume was 10 µL, consisting of 0.5 µM of each primer and 0.1 µM of probe. Standard cycling conditions were used [95°C for 10 minutes, (95°C for 10 seconds, 60°C for 30 seconds) x 50 cycles, 40°C for 40 seconds].

Each PCR experiment included a reaction in which template was replaced by water, and a reaction omitting reverse transcriptase as controls. Triplicate measurements were performed for all samples and standard curves. The percent coefficient of variation (%CV) for Cps was calculated for each sample. All samples for each gene were run on the same plate.

Endogenous control genes

The Roche Lightcycler 480 software (Roche diagnostics, Indianapolis, IN, USA) was used to perform 'absolute quantification' analysis of endogenous control gene expression using the standard curve second derivative maximum analysis method, which is a non-linear regression line method. A six point standard curve was used with a starting dilution of 1 and final concentration of 1.6E-03.

Five endogenous control genes (*AP3D1, C11H9ORF78, RPS9, RPS15A* and *PPIA*) were tested across all samples and their suitability determined using NormFinder and GeNorm (4, 64). Two novel endogenous control genes (*AP3D1* and *C11H9ORF78*) were derived from the microarray data as described previously (66). These two genes were the most stably expressed genes identified using microarray analysis. GeNorm identified *RPS9* and *RPS15A* as the best two genes for normalization (0.286 and 0.297 M values respectively with a combined average expression stability value of < 0.2). The next best gene was *AP3D1* with an M value of 0.263. Normfinder identified *RPS9* and *AP3D1* as the best combination of genes (0.008 and 0.009 stability values, respectively, with a combined stability value of 0.008). The next best gene was *RPS15A* with a stability value of 0.013. Based on these results the best 3 genes (*AP3D1*, *RPS9* and *RPS15A*) identified by both programmes were used for normalisation.

Relative quantification

The Roche Lightcycler 480 Software (Roche diagnostics, Indianapolis, IN, USA) was used to perform quantification using the 'advanced relative quantification analysis algorithm'. Three endogenous control genes (*AP3D1*, *RPS9* and *RPS15A)* were used to normalize the data, taking the geometric mean of the normalized ratio of target gene to each reference gene. A calibrator sample was then used as a control, whereby each calculated expression value was normalized to the calibrator sample.

The RT-PCR results were compared to those obtained using the microarray for four differentially expressed genes (*CCL2, GAPDH, IL8, MMP1, MSTN, PGST1,* and *TIMP1).* Correlation coefficients were calculated for all genes to compare the calculated gene expression data from qRT-PCR and the microarray data. Genes for RT-PCR were selected based on two criteria: biological significance and fold change (Table 1).

DNA methylation

DNA extraction and quality control

DNA was extracted from endometrial tissue using a Qiagen DNAeasy blood and tissue kit (QIAGEN catalogue# 69506). DNA quantity was determined by spectrophotometry using a Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). Genomic DNA integrity was

assessed by running it on a 0.8% Agarose Gel. Genomic DNA purity was assessed by the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Nanodrop technologies, Rockland, USA). DNA was considered to be good quality when there was a single clear band when run against a reference genomic DNA sample.

Sonication and immunoprecipitation of methylated regions

DNA was sonicated and immunoprecipitated as described previously (65). Briefly, 5 µg of DNA was sonicated, and the efficiency of sonication tested on the Agilent Bioanalyser. An ideal smear size ranged between 100-700 bp. The sonicated DNA was then immunoprecipitated using Anti 5 methyl cytidine (Cat. no: Eurogentec Ref: MECY_0500) bound to Pan Mouse IgG Dynabeads (Invitrogen Cat no: 110.41). DNA was eluted in 1% SDS solution at 65°C. Input DNA (whole cell extract- genomic DNA) and IP DNA were precipitated with 3M Acetate after a Phenol:chloroform (25:24:1) clean up, using heavy phase lock gels Cat. No. Eppendorf (ref # 2302830).

Labeling and microarray hybridization

A 400K custom designed Agilent microarray (2 x 400K array format, AMADID, Part no. and GEO platform are 027994, G4820A and GPL16270 respectively) was used for microarray hybridization. Microarray design has been described previously (65). Briefly, probes were designed to target all CpG islands in the bovine genome as designated by the UCSC genome browser (bosTau4.0). Input DNA was labeled with Cy3 and IP DNA with Cy5 fluorescent dye, using Agilent's Genomic DNA labeling kit (Cat. no. 5190-0453). Labeled samples were cleaned and concentrated using Microcon YM-30 columns (Millipore Cat. no. 42410). All of the labeled IP DNA (~ 3-4 µg) and 4 µg of labeled input DNA were used for microarray hybridization. Microarrays were hybridized for 40 hours at 65°C and then washed using a CGH wash buffer 1 and 2 for 5 minutes and 1 minute, respectively.

Microarray Scanning and Data Analysis

Microarray scanning and data analysis were conducted as described previously (65). Briefly, hybridized arrays were scanned at 2 µm resolution on an Agilent Technologies Scanner G2505C. Agilent Feature Extraction software (v10.5.1.1) was used for image data extraction and Agilent Genomic Workbench software was used for analysis. The centralization method

was used to normalize the data and methylation states were obtained using the BATMAN algorithm. The BATMAN algorithm determines the absolute methylation level for CpG dinucleotides contained within DNA fragments queried by probes across the microarray.

The methylation detection algorithm normalizes the log-ratios for each probe, based on its melting temperature, and returns the methylation status of a probe. Z-Scores and P-values were derived from the Gaussian data to give probabilities and confidence values for methylated and unmethylated probe populations. The algorithm then calculates a methylation log Odds, which gives the relative probability that a probe is more likely methylated than unmethylated.

Mapping of microarray probes to genes

Genomic co-ordinates for methylation probes were queried against RefSeq genes in the bovine genome (refFLAT file from UCSC bosTau4.0 (https://genome.ucsc.edu/)). Probes that mapped within the transcriptional boundaries or 10kb 5' of the transcriptional start site of a gene were annotated to that gene. Annotated methylation probes were then mapped to gene expression probes using the RefSeq accession as a common identifier.

Correlation between DNA methylation and gene expression

Correlations between DNA methylation and gene expression data for mapped probes were obtained using linear models, including infection status and tissue, and interactions in the model, giving a within group correlation between the 2 measurement types. A Benjamini Hochberg adjustment was made on the significance levels for the correlations.
The top 1,000 correlated genes were then compared to the lists of differentially expressed genes and enriched pathways. Methylation data have been submitted to NCBI gene expression omnibus (GSE60831).

Results

Milk production and metabolites

There were no differences detected in any of the milk production parameters measured or in the metabolites measured in this study (Table 1).

Progesterone and bacteriology

There were no differences in the bacteriology scores or concentration of progesterone in SCE and CON cows. Mean progesterone for CON and SCE cows was 1.4ng/mL and 3.4ng/mL respectively (SED 2.1, P = 0.4). The mean bacteriology scores for CON and SCE cows were 2.2 and 4.2 respectively (SED 1.6. P = 0.2). Four cows had an active corpus luteum, as evidence by progesterone concentrations above 1 ng/mL (3 SCE and 1 CON cow).

Histology

There was no significant difference ($P > 0.05$) between CON and SCE cows for any of the immune cells counted except for lymphocytes in the stratus spongiosum and stratus compactum ($P < 0.05$), where there were greater numbers in the SCE cows (Table 3). The number of neutrophils present in the uterus of CON and SCE cows did not differ in the epithelium (P = 0.18) or the stratum compactum (P = 0.45. Regression analysis revealed a positive linear (P = 0.001) association between bacteriology score and stratum compactum neutrophil numbers and a negative association ($P < 0.02$) between bacteriology score and epithelial lymphocyte and macrophage numbers. There was also a non-linear association among bacteriology score and epithelial neutrophils ($P < 0.0001$), stratum spongiosum lymphocytic foci (P = 0.003) and stratum spongiosum plasma cells ($P < 0.0001$) which were all greater with increased bacteriology score (Table 3).

Gene expression

For analysis of differential gene expression, cows with greater than 1 ng/mL of plasma progesterone were considered to have a functional corpus luteum and were omitted from the analysis. Three SCE and one CON cow were excluded based on this criterion. These samples were excluded because progesterone can markedly affect the immune response (29, 49). Analyses revealed 1,856 probes to be differentially expressed in cows with (SCE) versus without (CON) uterine inflammation ($P < 0.05$, Storey Multiple testing correction, supplementary Table 1). In addition, linear regression analysis revealed 2,976 probes that had significant correlations between gene expression and bacteriology score (Supplementary Table 1). Correlation of qRT-PCR and microarray data was 0.86 on average with all correlations above 0.7 for all genes tested (Table 1).

406 **Pathway Analysis**

407 **Comparison of SCE and CON cows**

408 Analysis of differentially expressed genes using GSEA identified several enriched pathways in
409 the SCE animals compared with the CON animals, the majority of which were related to the
410 innate and adaptive immune response pathways in the caruncular endometrium (Table 4
411 and 5). There were no pathways enriched in the CON group. Several of the genes in the
412 enriched pathways displayed large expression differences between the two groups while
413 the enriched pathways in the intercaruncular endometrium showed relatively small
414 differences (data not presented) in gene expression between the two groups (Table 5).

415 **Gene expression associations with bacteriology score**

416 DAVID analysis revealed several pathways enriched in genes with a significant association
417 among gene expression and bacteriology score (Table 6). The majority of pathways were
418 related to both the innate and adaptive immune response. Gene ontology analysis using
419 DAVID revealed several biological processes that were enriched in the genes that had
420 expression data correlated to the bacteriology score (Table 7). The top three gene ontology
421 terms were defense response, inflammatory response and the innate immune response
422 (Tables 6 and 7).

423 **Correlation of DNA methylation and gene expression data**

424 Transcription data from RNA extracted from the same tissues that DNA was extracted from
425 for this study were used to identify genes potentially regulated by DNA methylation. No
426 correlations were significant after correction for multiple hypothesis testing ($P < 0.05$).
427 Therefore, the 1,000 most statistically significant data points were used for subsequent
428 analysis (688 were unique genes). Forty five percent of these DNA methylation probes were
429 negatively correlated with the signal from gene expression probes while 55% were positively
430 correlated. When this gene list was compared with gene expression in the same tissue, 13%
431 of these were differentially expressed between SCE and CON animals and 10%
432 demonstrated positive correlations with bacteriology score (Supplementary Table 2). The
433 microarray data have been submitted to the NCBI gene expression omnibus (GSE58794).

Discussion

Genes of the innate immune system were upregulated in cows with sub-clinical endometritis and their expression was positively correlated with bacteriology score. Several pathways involved in the innate immune response were enriched in SCE cows and there was significant enrichment for inflammatory and immune response pathways and GO terms in genes demonstrating a significant correlation among bacteriology score and gene expression. Consistent with activation of toll like receptors (TLR) by microorganisms present in the uterus, there was enrichment for the TLR signaling pathway, including increased expression of the transcription factor *NFKB1*, the pro-inflammatory cytokines *IL1A* and *IL1B* and downstream chemokines, cytokines, and acute phase and antimicrobial proteins in the endometrium of SCE cows. The endometrium expresses high levels of pattern recognition receptors (PRR), such as TLR, which recognize pathogen-associated molecular patterns (PAMPS) present on microorganisms (1, 26). Binding of those PRR to PAMP initiates host defense mechanisms, such as production of inflammatory cytokines and chemokines (26, 41). These, in turn, promote recruitment of immune cells to the site of infection, production of acute phase and antimicrobial proteins, and activation of the adaptive immune system (2, 3) in cows with sub-clinical endometritis. It is well established that sub-clinical endometritis (defined by elevated PMN) during the postpartum period is associated with poor reproductive performance (27, 39, 40). Gene expression profiles in cows with subclinical endometritis in this study indicate that the immune response is activated, potentially resulting in a local pro-inflammatory environment in the uterus. If this period of inflammation is prolonged, it could result in tissue damage or failure to complete involution of the uterus and this may create a sub-optimal environment for future pregnancy. Inflammation in the uterus during embryo development could result in failure to create immune tolerance to the embryo and therefore pregnancy may be lost due to immune rejection of the embryo.

Antimicrobial proteins and the acute phase response

During parturition the anatomical defense barriers of the uterus are compromised, making it vulnerable to infection from microorganisms (5, 43, 59). Bacterial invasion of the uterus triggers local immune responses including expression of pro-inflammatory cytokines that

trigger epithelial and recruited innate immune cells to produce antimicrobial peptides (AMP) and acute phase proteins (12). Consistent with this, several antimicrobial peptides (*LAP, TAP, DEFB1, DEFB10, DEFB103B, DEFB7*) and acute phase proteins (APP), including *SAA3, LBP* and the complement gene *CFB,* had greater gene expression in SCE cows. Furthermore, the expression of several complement genes (*CFB, C1QA, C1R, C1S* and *C2*) was positively correlated with bacteriology score. Additionally, gene expression of the S100 family of calcium binding proteins that possess antimicrobial properties and are a marker of inflammation, were upregulated in the endometrium of SCE cows (*S100A12, S100A14, S100A3, S100A4, S100A8, S100A9*). Antimicrobial proteins are an important part of mucosal immunity and, along with mucins, they prevent attachment and colonization of the uterus with microorganisms (13). Several of these molecules act as opsonins for macrophages and neutrophils, enhancing phagocytosis efficiency (31). The APP LBP binds to the bacterial endotoxin, lipopolysaccharide (LPS) and presents it to membrane bound CD14. This complex can then interact with TLR4, triggering a pro-inflammatory reaction (69). Furthermore, the APP SAA enhances neutrophil respiratory burst and macrophage TNF-α and IL-10 production (57). Additionally, SAA binds to high density lipoprotein (HDL), becoming the major apolipoprotein of HDL. This results in reduced reverse cholesterol transport. During the acute phase response, when there is an increase in SAA, secretory phospholipase also increases. This phospholipase can hydrolyse surface phospholipids on HDL increasing delivery of cholesterol to peripheral tissues for cellular repair (48). The availability of lipoproteins during early lactation is limited by the ability of the liver to assemble and release them (28, 38, 56). Limited availability of lipoproteins for tissue repair could have negative consequences for uterine recovery postpartum. In addition, lipoproteins can bind endotoxin from bacteria, competing with immune cells for endotoxin binding (30). Binding of endotoxin by HDL and delivery to the liver for breakdown could be an important mechanism to both clear infection and limit the pro-inflammatory reaction to infection.

Up-regulation in the genes of acute phase and antimicrobial proteins in SCE cows, despite no difference in the bacteriology score, may be due to inappropriate continuation of a pro-inflammatory response to cleared bacteria. Alternatively, given the culture dependent measurements undertaken in this study, there could be undetected differences in uterine microbial diversity underlying the etiology of sub-clinical endometritis. In support of this,

studies utilizing culture-independent methods have reported that the microbiome of cows with endometritis or metritis differs to that of healthy cows, particularly at days 34-36 postpartum (55). Furthermore, cows with a greater prevalence of particular bacteria at day 35 postpartum were less likely to be pregnant by day 200 postpartum (36). Expression of acute phase and antimicrobial genes in the absence of bacteriology differences may be indicative of a failure to control the pro-inflammatory state in some cows; however, further investigation of the microbiome of the post-partum uterus is warranted.

Recruitment of innate immune cells

Cows with sub-clinical endometritis were actively recruiting immune cells to the uterus through the expression of chemotactic genes. The chemokine signaling pathway was enriched in SCE cows and there was enrichment for the chemokine signaling pathway in genes correlated with bacteriology score. Increased expression of *IL-8* and *CXCL6,* chemotactic factors for recruitment of neutrophils, along with the immune cell surface marker *PTPRC* in SCE cows, is consistent with the diagnosis of sub-clinical endometritis (17, 62). Furthermore, *IL-1B* expression stimulates expression of both *CXCL6* and *IL-8* (54) and was upregulated in SCE cows and positively correlated with bacterial load. There was also a positive correlation among bacteriology score and gene expression of *IL-8* and *CXCL6,* indicating that with increased bacterial load, the uterus attempts to recruit more neutrophils to resolve the infection. Further, histological analysis revealed a positive association among bacteriology score and tissue neutrophil number, as well as a trend for increased neutrophils in SCE cows. Additionally, there was an increase in *CD14* expression associated with bacteriology score which suggests along with increased bacterial load that there may have been an increase in the recruitment of other immune cell types, such as monocytes to the uterus (46). Consistent with this, there was upregulation of monocyte chemoattractants. The chemokine CCL2 is a strong chemoattractant for monocytes and CXCL6 attracts both monocytes and neutrophils (19). Both of these genes were upregulated in SCE cows, with *CXCL6* expression positively correlated with bacterial load. Once monocytes reach the site of infection, they differentiate into tissue macrophages or dendritic cells (20, 61). Therefore, it might be expected that there would be greater numbers of tissue macrophages with increased bacteriology score. However, there was a negative linear trend for macrophage number with increased bacteriology score. Lack of

macrophage numbers despite upregulation of monocyte chemoattractants may indicate monocytes were unresponsive to signals and could explain why some cows have a prolonged inflammatory state. Alternatively, with the presence of more macrophages there should be greater efficiency in clearing pathogens.

Natural killer cells (NK cells) may be activated in the uterus of SCE cows and there was a positive association between NK cell pathway gene expression and bacteriology score. The NK cell mediated cytotoxicity pathway was enriched in SCE cows and in genes correlated with bacteriology score. Furthermore, histological assessment of the uterus revealed greater lymphocytes in the stratum compactum of SCE cows, which may be indicative of NK cell enrichment. There was upregulation of the $CD16^-$ NK cell chemoattractant and co-stimulator of proliferation *CCL19* in the uterus of SCE cows and the $CD16^+$ chemoattractant *IL-8* (46, 48). Genes in the NK cell mediated cytotoxicity pathway that were upregulated in SCE cows include the NK cell activating receptors (*FCGR3A* and *NCR3*), along with genes encoding cytotoxic granules (*PRF1* and *GZMB*), and intracellular signaling proteins (*FCER1G*, *ZAP70*, *syk*, and *VAV-1*) required for the release of those cytotoxic granules (8). NK cells link the innate and adaptive immune response and can be both disease controlling or promoting, depending on the subset and activation state (70). Further research investigating the types of NK cells present in the uterus of cattle is required to unravel their potential role in subclinical endometritis.

Adaptive immune response

The adaptive immune response is activated in cows that fail to eliminate pathogens effectively via the innate immune response. The hypothesis that the adaptive immune response is active in SCE cows and in cows with a high bacteriology score is supported by pathway analysis. Several pathways of the adaptive immune response were enriched in SCE cows and were enriched in genes that had a positive correlation with bacteriology score. Furthermore, there were greater numbers of lymphocytes in SCE cows, and lymphocytic foci and plasma cell numbers were positively associated with bacteriology score. In early lactation, dairy cattle are prone to metabolic stress associated with negative energy balance. During this period, the immune system is suppressed; for example, there is evidence that neutrophil function is impaired (18, 23, 37). Impaired function of the innate

556 immune system would likely result in delayed elimination of pathogens, prolonging infection
557 and, consequently, a harmful inflammatory state. While the innate immune system is the
558 first line of defense against pathogens, a co-ordinated response from both the innate and
559 adaptive immune system is required for efficient elimination of pathogens. Cows
560 experiencing a period of severe negative energy balance at 2 weeks postpartum had more
561 stromal lymphocytic foci (63). While we could not determine if higher bacteriology score
562 was the result of inefficiency in the innate or adaptive immune system, without an efficient
563 innate immune response to contain infection there would be a higher likelihood of adaptive
564 immune response activation, and prolonged inflammation.

565 **DNA methylation**

566 There was no significant correlation among DNA methylation and gene expression. To
567 identify genes for future study, the 1,000 most significant correlations were compared with
568 genes in the most enriched pathways. There were very few genes that were in both the top
569 1,000 correlations and in the most significantly enriched pathways. Furthermore, very few
570 genes that were differentially expressed between SCE and CON cows or those that were
571 correlated with bacteriology score were in the top 1,000 correlations for DNA methylation.
572 Interestingly, most of these genes were positively correlated with DNA methylation rather
573 than negatively correlated. Of particular interest is the positive correlation among *SAA3*
574 gene expression and promoter DNA methylation. This gene is up-regulated in response to
575 infection, in agreement with the current study; however, treatment with a DNA methylation
576 inhibitor results in greater expression of *SAA3* in response to LPS (22), suggesting that DNA
577 methylation in the promoter of *SAA3* may result in decreased responsiveness to infection.
578 The innate immune response to infection is regulated by DNA methylation and histone
579 acetylation (53) and variation in DNA methylation could explain differing responses to post-
580 partum infection in dairy cows (22). Further, loss of epigenetic marks, such as acetylation of
581 histone tails that enable transcriptional activation of pro-inflammatory genes, has been
582 proposed as a mechanism to prevent excessive inflammation (15). Removal of acetyl tags
583 from histone tails results in suppression of gene expression; treatment of cells with histone
584 deacetylase inhibitors (maintaining acetyl tags on histones) results in increased NFKB1
585 stimulated expression of inflammatory genes. While there was a lack of detectable
586 association between DNA methylation and gene expression in this study, there are several

studies using a similar genome-wide approach that provide compelling evidence supporting the role of DNA methylation in regulating endometrial function. For example, in women there is evidence for reproductive cycle stage dependent DNA methylation associated with transcription (25). Genome-wide DNA methylation is altered in women with endometriosis (14, 44). Furthermore, in the cow, DNA methylation is negatively correlated with gene expression in the endometrium during pregnancy (65). DNA methylation is very cell specific (16, 24) and given that the samples used in this study were from a biopsy sample it is likely that true correlations among DNA methylation and gene expression were not detected because of the 'noise' created by the many different cell types present. Future research should focus on developing techniques for isolation of specific cell types from biopsy samples such as laser capture microdissection. DNA methylation is one of the mechanisms used by cells to create unique expression profiles and functions among different types of cells. Therefore, establishing which cell types express key genes and how DNA methylation patterns drive these changes in a single cell type will improve our understanding of the biology of post-partum uterine infection.

Conclusion

We hypothesized that aberrant DNA methylation may be involved in the sub-fertility associated with post-partum uterine inflammation. While we were unable to detect any association among gene expression and DNA methylation we did find that the expression of genes regulating the immune response were greater in cows with sub-clinical endometritis. The lack of differences detected in bacteria present in the uterus suggests that some cows may fail to control inflammation even when there are no bacteria present. Further, lack of difference in macrophage number in the uterus despite greater expression of macrophage attracting chemokines suggests that these cells may become unresponsive to these signals, delaying tissue repair and prolonging inflammation. If this inflammation is sustained into the breeding period it could result in a sub-optimal environment for embryo development and potentially failure to create local immune tolerance to the embryo.

Authors' contributions

CGW was involved in experimental design, performed the experimental work and statistical analysis, and wrote the manuscript. SM designed the animal trial and undertook sample collection. HH performed the bacteriology experiments. SMcD was involved in experimental design of the animal trial and sample collection. JRR was involved in experimental design of the animal trial and critical analysis of the manuscript. CRB was involved in experimental design of the animal trial and critical analysis of the manuscript. MDM was the project leader and was involved in experimental design, critical analysis of the manuscript and general supervision of the project.

All authors read and approved the final manuscript.

Acknowledgements

This work was supported by funding from New Zealand dairy farmers through DairyNZ Inc. (New Zealand; Project AN808) and the Ministry of Business, Innovation and Employment (UOAX0814). The authors acknowledge the DairyNZ farm and technical staff who completed the animal work. The authors thank the staff at the AgResearch Abattoir (AgResearch, Ruakura) for their support of this research. The authors acknowledge the statistical advice and assistance of Barbara Dow and Barbara Kuhn-Sherlock. The authors acknowledge Kelly Buckle (IVABS, Massey University, Palmerston North, New Zealand) who undertook cytological examination of slides. The authors acknowledge Michael Hardcastle at Gribbles Veterinary Pathology for histological analysis.

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Table Legends

Table 1 qRT-PCR confirmation of microarray. Primer sequences and UPL probe used for qRT-PCR assays.

Table 2 Milk production and metabolite data

ANOVA was used to determine the effect of health status (SCE vs CON) on measurements of milk production and metabolites. Data is presented at Least square means (LSMeans).

Table 3 Histological assessment of uterine tissues. Results for the analysis of immune cells present in the uterine sample. ANOVA, linear regression and quadratic regression were used to determine the effects of health status (SCE vs CON) and bacteriology score, on the number of immune cells present in the epithelia, stratus compactus, and stratus spongiosum. Immune cells detected included, lymphocytes, neutrophils, eosinophils, macrophage, and plasma cells. The presence of lymphocytic foci was also assessed.

Table 4 Enriched pathways identified using GSEA for the comparison of SCE and CON cows in caruncular (CAR) and intercaruncular (ICAR) endometrium. Enrichment score (ES), normalised enrichment score (NES), false discovery rate (FDR), family wise error rate (FWER).

Table 5 Genes in the enriched pathways identified using GSEA for the comparison of SCE and CON cows in caruncular (CAR) endometrium. Enrichment score (ES), normalised enrichment score (NES), false discovery rate (FDR), family wise error rate (FWER). Genes (human gene symbols) with fold changes greater than 1.5 or less than -1.5 are in bold.

Table 6 Pathways identified as enriched using DAVID for genes that have expression associated with bacteriology score. The number of genes in the pathway (count), Percentage of the genes in the pathway (%), total number of genes in the list (LT), number of genes in the background list in the pathway (PH), number of genes in the background list in any ontology term. The ratio of the percentage of genes in the pathway to the percentage of genes in the background that are in that pathway (fold enrichment).

Table 7 Enriched GO Terms identified using DAVID in genes that have expression associated with bacteriology score. The number of genes in the term (count), Percentage of the genes in the term (%), total number of genes in the list (LT), number of genes in the background list in the term (PH), number of genes in the background list in any ontology term. The ratio of the percentage of genes in the term to the percentage of genes in the background that are in that term (fold enrichment)

Supplementary Table 1

Gene expression analyses revealed 1,856 probes to be differentially expressed in cows with or without uterine inflammation ($q<0.05$, Storey multiple testing correction). Additionally, 2,976 probes had gene expression correlated with bacteriology score ($P<0.05$, Benjamini-Hochberg multiple testing correction). The coefficient of determination (r^2) describes the proportion of the variance in gene expression that is predicted from the bacteriology data.

Supplementary Table 2

DNA methylation correlations with gene expression. As there were no significant differences detected after multiple hypothesis testing, the 1,000 most significant correlations are

881 presented. Forty five percent of these DNA methylation probes were negatively correlated
882 with signal from gene expression probes while 55% were positively correlated. When this
883 gene list was compared with gene expression on the same tissue, 13% of these were
884 differentially expressed between SCE and CON animals and 10% demonstrated positive
885 correlations with bacteriology score.

Table 1

Primer Sequences 3' to 5'

	Forward	Reverse	Probe	Exon boundary	Correlation	P-value
CCL2	CCAGCAGCAAGTGTCCTAAA	GCTTGGGGTCTGCACATAAC	61	Exon2/3	0.8	0.04
GAPDH	GAAGCTCGTCATCAATGGAAA	CCACTTGATGTTGGCAGGAT	9	Exon2/3	0.9	0.04
IL8	CTGGCTGTTGCTCTCTTGG	CTGTACTCATTCTTGACAGAACTGC	15	Exon1/2	0.8	0.04
MMP1	TGGGAAAACCTATTTCTTTGTTG	CCTGCATCCATGGACTGTTTA	67	Exon7/8	0.7	0.04
MSTN	TCTTGCTGTAACCTTCCCAGA	TTGGTGTGTCTGTTACCTTGACTT	2	Exon2/3	0.8	0.04
PGST2	CCCTATGAATCATTTGAGGAACTT	TGTCTCCATAGAGCGCTTCTAACT	46	Exon8/9/10	0.8	0.05
TIMP1	TATGCTGCTGGTTGTGAGGA	CTCTGGAACCCCTTGTCAGA	100	Exon4/5	0.7	0.15
Reference genes						
AP3D1	AGTGCTCATCTCCCTGTCATC	CCTCAATTTCTGAACGCAGAG	89	Exon8/9/10/11		
C11H9ORF78	CATTTTCCGCAGAAACCAAC	CCGCTTCTTCAGTTCTGTCTC	153	Exon4/5		
RPS15A	TCAGCCCTAGATTTGATGTGC	GCCAGCTGAGGTTGTCAGTA	32	Exon4/5		
PPIA	GTCAACCCCACCGTGTTCT	TTCTGCTGTCTTTGGAACTTTG	152	Exon1/2/3		
RPS9	TGAGGATTTCTTGGAGAGACG	ATGTTCACCACCTGCTTGC	138	Exon4/5		

Table 2

Parameter	CON	SCE	SED	P-Value
Total Protein (g/L)	77.17	82.17	3.96	0.24
Albumin (g/L)	37.50	36.83	2.05	0.75
Globulin (g/L)[1]	39.67	45.33	3.19	0.11
Albumin:Globulin ratio	0.95	0.83	0.09	0.23
Magnesium (mmol/L)	0.70	0.67	0.09	0.70
Non-esterified fatty acids (mmol/L)	1.70	1.50	0.26	0.46
GDH (IU/L)[2]	28.33	24.33	7.85	0.62
ASAT (IU/L)[3]	87.50	78.50	6.61	0.20
Milk yield (kg/d)[4]	23.51	22.91	1.74	0.74
Milk Fat (%)[5]	4.35	4.40	0.31	0.90
Milk crude protein (%)[5]	3.48	3.59	0.09	0.22
Total solids (%)[5]	7.83	7.99	0.36	0.68
Somatic cell count*[5]	45.69	47.33	12.60	0.60
Live weight (kg)[6]	477.39	494.56	26.08	0.53
Average body condition (units)[6]	4.36	4.28	0.22	0.71

[1] Globulin is calculated when albumin is subtracted from total protein

[2] GDH = glutamate dehydrogenase

[3] ASAT = aspartate aminotransferase

[4]LSMean of daily measures taken over 26 to 31 days (varies across cows)

[5] LSMean of measures taken at weekly herd tests (range from 3 to 4 herd tests per cow)

[6] LSMean of 3 measurements taken post-calving at weeks 1, 2 and 3

* P-Values are based on analysis of Log10 transformed data

Table 3

		ANOVA for effect of Status					Linear Regression			Quadratic Regression				
Location	**Cell type**	**CON**	**SCE**	**SEM**	**SED**	**P-value**	**Intercept**	**Bacteriology**	**P-Value**	**Intercept**	**Bacteriology**	**Bacteriology^2**	**Probability**	**P-Value**
Epithelia	Neutrophil	2.03	7.17	2.50	3.54	0.18	-1.88	2.05	<0.0001	1.29	-0.67	0.32	0.01	<0.0001
	Lymphocyte	10.51	11.26	2.83	4.00	0.86	15.96	-1.60	0.02	17.68	-3.08	0.17	0.46	0.05
	Macrophage	0.33	0.02	0.17	0.24	0.22	0.28	-0.03	0.49	0.29	-0.04	0.00	0.99	0.80
	Eosinophil	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.52	0.00	0.00	0.00	0.27	0.46
Stratum compactum	Neutrophil	12.19	24.07	10.66	15.08	0.45	-5.84	7.57	<0.001	-2.25	4.49	0.36	0.61	0.01
	Lymphocyte	49.81	79.96	9.63	13.62	0.05	61.16	1.18	0.71	72.12	-8.21	1.09	0.40	0.65
	Macrophage	12.27	10.40	3.09	4.37	0.68	16.07	-1.50	0.05	14.80	-0.41	-0.13	0.66	0.15
	Eosinophil	1.69	0.27	1.14	1.61	0.40	0.42	0.18	0.58	-0.94	1.34	-0.14	0.30	0.48
	Plasma cells	2.17	79.70	31.65	44.76	0.11	-35.03	23.99	<0.005	32.18	-33.57	6.70	<0.001	<0.001
	Lymphocytic foci	0.28	2.26	0.83	1.18	0.12	1.80	-0.17	0.51	1.88	-0.23	0.01	0.95	0.81
Stratus Spongiousum	Lymphocytic foci	0.28	3.67	1.04	1.47	0.04	0.00	0.62	0.05	2.66	-1.66	0.27	0.01	<0.005

Table 4

KEGG pathways enriched in SCE CAR

KEGG pathway	ES	NES	FDR	FWER
Primary immunodeficiency	0.66	2.20	<0.01	<0.01
Natural killer cell mediated cytotoxicity	0.51	2.15	<0.01	<0.01
Proteasome	0.58	2.13	<0.01	<0.01
Systemic lupus erythematosus	0.53	2.07	<0.01	<0.01
Amino sugar and nucleotide sugar metabolism	0.55	2.06	<0.01	0.01
RNA polymerase	0.63	2.02	<0.01	0.01
Chemokine signaling pathway	0.44	2.03	<0.01	0.01
T cell receptor signaling pathway	0.48	2.04	<0.01	0.01
Intestinal immune network for IGA production	0.61	1.99	<0.01	0.02
Parkinsons disease	0.43	1.95	<0.01	0.03
Citrate cycle tca cycle	0.58	1.93	<0.01	0.04
Spliceosome	0.43	1.91	0.01	0.05
Cytosolic dna sensing pathway	0.53	1.90	0.01	0.06
Pyrimidine metabolism	0.45	1.88	0.01	0.07
Oxidative phosphorylation	0.42	1.87	0.01	0.09
B cell receptor signaling pathway	0.46	1.83	0.01	0.13
Alzheimers disease	0.39	1.82	0.01	0.15
Autoimmune thyroid disease	0.61	1.81	0.01	0.17
Purine metabolism	0.39	1.79	0.01	0.19
Glycosphingolipid biosynthesis lacto and neolacto series	0.62	1.76	0.02	0.25
N glycan biosynthesis	0.49	1.75	0.02	0.28
Huntingtons disease	0.37	1.75	0.02	0.28
Graft versus host disease	0.60	1.74	0.02	0.31
Prion diseases	0.53	1.72	0.02	0.34
Fc epsilon ri signaling pathway	0.43	1.67	0.03	0.48
Nod like receptor signaling pathway	0.44	1.67	0.03	0.50
Glycolysis gluconeogenesis	0.45	1.66	0.04	0.54
Protein export	0.52	1.64	0.04	0.59
Cytokine cytokine receptor interaction	0.35	1.63	0.04	0.62
Enriched in SCE ICAR				
Parkinsons disease	0.59	2.76	<0.01	<0.01
Oxidative phosphorylation	0.57	2.66	<0.01	<0.01
Alzheimers disease	0.53	2.53	<0.01	<0.01
Citrate cycle tca cycle	0.71	2.38	<0.01	<0.01
Huntingtons disease	0.47	2.29	<0.01	<0.01
Propanoate metabolism	0.65	2.18	<0.01	<0.01
Valine leucine and isoleucine degradation	0.59	2.16	<0.01	0.01
Cardiac muscle contraction	0.56	2.19	<0.01	0.00
Steroid hormone biosynthesis	0.63	2.00	<0.01	0.02
Fatty acid metabolism	0.55	1.97	<0.01	0.03
Glycine serine and threonine metabolism	0.61	1.97	<0.01	0.03
Protein export	0.60	1.94	0.01	0.05
Pyruvate metabolism	0.54	1.89	0.01	0.08
Proteasome	0.48	1.85	0.01	0.11
N glycan biosynthesis	0.51	1.84	0.01	0.11
Retinol metabolism	0.56	1.83	0.01	0.13
Glutathione metabolism	0.48	1.78	0.01	0.18
Arginine and proline metabolism	0.46	1.76	0.02	0.21
Cysteine and methionine metabolism	0.51	1.70	0.03	0.33
Biosynthesis of unsaturated fatty acids	0.55	1.66	0.04	0.44

Table 5

Top 10 pathways enriched in SCE CAR

KEGG Pathway	ES	NES	FDR	FWER
primary immunodeficiency	0.66	2.20	<0.001	<0.001
Up-regulated genes				
ICOS, TNFRSF13B, ZAP70, CD3E, CD79A, LCK, CD3D, TNFRSF13C, CD40, IL7R, TAP1, BLNK, PTPRC, CD8A, BTK, RFX5, TAP2, CD4, IKBKG *, CD8B, RFX5, TAP2, IL2RG, ADA, IKBKG*				
Down-regulated genes				
RFXANK, RFXAP, UNG				
Natural killer cell mediated cytotoxicity	0.51	2.15	<0.001	<0.001
Up-regulated genes				
CD48, TNF, GZMB, RAC2, PIK3CG, SH2D1A, HCST, LCK, LAT, ZAP70, PRF1, VAV1, LCP2, KLRK1, CD244, SYK, PIK3CD, HLA-A, CD247, ITGAL, PTPN6, FASLG, ITGB2, FAS, PTK2B *, IFNAR1, NCR3, MAP2K1, CASP3, GRB2, IFNAR2, FYN, NFATC1, FCGR3A, FCER1G, IFNGR2, TYROBP, PIK3CB, SHC1, MAP2K2, NFATC3, NRAS, BID, PTPN11, KRAS, ARAF, PPP3CA, RAC1, PPP3R1, IFNGR1, TNFSF10*				
Down-regulated genes				
PRKCA, PPP3CC, SOS2, MAPK3, RAF1, PIK3R1, NFAT5, PPP3CB, PLCG1, PIK3CA, PAK1, VAV3, , RAC3, PIK3R2, ***SHC2, PIK3R3***				
Proteasome	0.58	2.13	<0.001	<0.001
Up-regulated genes				
PSMB9 *, PSMB8, PSMD14, PSMA2, PSMD3, PSMD8, PSMA7, PSMB10, PSMA1, PSMB1, PSMD1, PSME2, PSMB6, PSMB2, PSMA6, PSMC4, PSMB4, PSMA5, PSMA4, PSMB3, PSMD6, POMP, , PSMC5, PSMA3, PSME1, PSMD7, PSMC3, PSMB5, PSMC6, PSMB7, PSMD11, PSMD12, PSMC1, SHFM1*				
Down-regulated genes				
PSME4, PSMD4, PSMF1, PSMD13,				
Systemic lupus erythematosus	0.53	2.07	0.001	0.004
Up-regulated genes				
C3, CD40, TNF, HIST1H2AH, C2, C1QB, C1QC, C1QA, HIST1H2AG, C4A, HLA-DRA, FCGR1A, HIST1H2BN *, CD86, HLA-DMB, IL10, ACTN1, SNRPD1, HIST1H2BD, HLA-DOA, FCGR3A, ACTN3, SSB, H2AFZ, ACTN4, SNRPB, SNRPD3, C1S, HLA-DMA, HIST2H2AC, H3F3A, H2AFX, H2AFV, TROVE2, HIST1H3A, H2AFY*				
Down-regulated genes				
C5, H2AFY2, TRIM21, ***ACTN2***				
Amino sugar and nucleotide sugar metabolism	0.55	2.06	0.001	0.005
Up-regulated genes				
NANS, GNPNAT1 *, UGDH, GALE, TSTA3, PGM3, PMM2, UXS1, HEXB, GMDS, GFPT2, GMPPA, GFPT1, CMAS, CYB5R1, GPI, MPI, GALK2, GALK1, UAP1, UGP2, PMM1, GALT, GNPDA2, NANP, NAGK*				
Down-regulated genes				
GNPDA1, HEXA, PGM1, AMDHD2, CYB5R3, FPGT, GCK, PGM2, FUK, NPL				
RNA polymerase	0.63	2.02	0.001	0.009
Up-regulated genes				
POLR3G *, POLR2L, POLR2E, POLR1B, POLR3D, POLR3K, POLR2C, POLR1C, POLR1A, POLR3B, POLR2K, POLR2F, POLR2B, POLR2G, POLR2I, POLR2J, POLR2D, POLR3F*				
Down-regulated genes				
POLR3C				
Chemokine signaling pathway	0.44	2.03	0.002	0.009
Up-regulated genes				
CXCL5, IL8, CXCL2, CXCR5, CCL19, CCL20, CCR7, CCL2, RAC2, CCL24, PIK3CG, CCL4, CCL8, CCL5, CCL28, LYN, CXCR6, ITK, CCR1, RASGRP2, VAV1, CCR5, PRKCB, HCK, PIK3CD, GNG3, GNAI2, CXCL16, PTK2B, ARRB1 *, CXCR4, PRKCD, STAT3, GNG2, ADRBK1, MAP2K1, CSK, NFKBIB, ADCY7, GRB2, NFKB1, GNG5, GNG10, ROCK2, RAP1A, STAT5B, GNB5, GNB2, PRKX, ADCY4, RAP1B, GNAI3, GNB1, IKBKG, PIK3CB, XCL2, SHC1, WASL, RHOA, CXCL10, ARRB2, AKT1, NCF1, NRAS, NFKBIA, IKBKB, GNG12, KRAS, CXCL14, RAC1, STAT2, PRKCZ, ROCK1, PRKACB, BCAR1, PREX1. FGR. CHUK. PRKACA. GSK3A*				
Down-regulated genes				
RELA, SOS2, JAK2, PLCB3, GNB3, MAPK3, ADRBK2, RAF1, GNGT2, GSK3B, CRK, CCL25, CXCL9, PIK3R1, ADCY3, STAT1, GNB4, PIK3CA, FOXO3, GNG7, CCL21, ADCY6, PARD3, GNAI1, PAK1, VAV3, TIAM1, ADCY2, CCL16, PIK3R2, CCL14, GNG11ADCY8, ***ADCY5, PLCB4, CCL11, CX3CR1, SHC2, PIK3R3***				
T cell receptor signaling pathway	0.48	2.04	0.002	0.009
Up-regulated genes				
ICOS, MAPK13, TNF, CD3D, PRKCQ, CD3G, PIK3CG, PTPRC, ITK, CD3E, LCK, LAT, ZAP70, VAV1, LCP2, PIK3CD, CD247, CD8A, CARD11 *, CD8B, MAP2K1, IL10, NFKBIB, GRB2, NFKB1, FYN, NFATC1, PDCD1, MALT1, NFKBIE, IKBKG, CDK4, NCK1, PIK3CB, MAP2K2, RHOA, NFATC3, AKT1, NRAS, MAPK14, NFKBIA, IKBKB, KRAS, MAP3K7, PPP3CA, BCL10. MAPK12, PPP3R1, CHUK. CD4,*				
Down-regulated genes				
RELA, TEC, PPP3CC, PAK6, SOS2, FOS, MAPK3, CBLB, RAF1, MAPK9, GSK3B, PIK3R1, NFAT5, CBLC, NCK2, PAK4, PPP3CB, PDK1, PLCG1, PIK3CA, MAP2K7, PAK1, VAV3, PAK3, PIK3R2, ***JUN, PIK3R3***				
Intestinal immune network for IGA production	0.61	1.99	0.002	0.018
Up-regulated genes				
PIGR, ICOS, TNFRSF13C, CD40, CCL28, ITGB7, HLA-DRA *, CXCR4, CD86, HLA-DMB, IL10, IL15RA, HLA-DOA, MADCAM1, HLA-DMA, TNFSF13, LTBR*				
Down-regulated genes				
CCL25				
Parkinsons disease	0.43	1.95	0.003	0.027
Up-regulated genes				
UBE2J1, CYCS *, SLC25A5, UQCRC1, COX6B2, ATP5B, UBE2G2, CASP3, ATP5G3, NDUFB5, ATP5G1, NDUFA2, COX5A, UQCRB, NDUFA4, ATP5C1, UQCRC2, NDUFA5, NDUFS6, NDUFA1, NDUFV1, UQCRQ, COX7B, UBE2L3, NDUFB2, VDAC1, NDUFB1, NDUFB8, NDUFS8, SLC25A4, UBE2G1, NDUFS1, COX7A2, CYC1, NDUFB4, UQCR11, NDUFC1, UBA1, COX7C, NDUFB9, UQCRFS1, NDUFA10, ND6, NDUFS7, UQCR10, NDUFA9, PARK2, NDUFAB1, UBE2J2, NDUFA7, NDUFB7, ATP5J, SDHC, COX5B, NDUFB6, NDUFA4L2, ATP5O, NDUFV2, VDAC3, ATP5F1, SEPT5, PPID, NDUFB3, ATP5A1, PARK7, VDAC2, COX7A1, UCHL1, COX6C, NDUFC2*				
Down-regulated genes				
COX4I1, NDUFS4, TH, PINK1, COX4I2, ATP5H, SDHB, COX2, UBE2L6, NDUFA8, NDUFS5, NDUFA3, HTRA2, ATP5D, NDUFB10, NDUFS3, UBA7, GPR37, SNCA, NDUFS2, ND2, UQCRH, NDUFA6, COX7A2L, LRRK2, CASP9, ND4L, ATP5G2, SLC18A2, ND4, ND5, ATP6, ***CYTB, COX3, ND1, SNCAIP***				

Table 6

KEGG Pathway	Count	LT	PH	PT	%	P-Value	Fold Enrichment	Fisher Exact
Fc epsilon RI signaling pathway	20	492	48	2862	1.1	1.70E-04	2.4	5.00E-05
Ubiquitin mediated proteolysis	25	492	78	2862	1.4	2.00E-03	1.9	8.50E-04
B cell receptor signaling pathway	20	492	58	2862	1.1	2.60E-03	2	1.00E-03
Neurotrophin signaling pathway	25	492	85	2862	1.4	6.70E-03	1.7	3.30E-03
Focal adhesion	34	492	129	2862	1.9	8.70E-03	1.5	4.90E-03
Adherens junction	16	492	47	2862	0.9	9.30E-03	2	3.70E-03
Complement and coagulation cascades	15	492	43	2862	0.8	9.80E-03	2	3.70E-03
Chemokine signaling pathway	32	492	124	2862	1.8	0.02	1.5	8.70E-03
Regulation of actin cytoskeleton	33	492	130	2862	1.8	0.02	1.5	0.01
NOD-like receptor signaling pathway	13	492	39	2862	0.7	0.03	1.9	0.01
Renal cell carcinoma	15	492	48	2862	0.8	0.03	1.8	0.01
Pathways in cancer	47	492	206	2862	2.6	0.03	1.3	0.02
Methane metabolism	4	492	5	2862	0.2	0.04	4.7	3.70E-03
Pancreatic cancer	14	492	46	2862	0.8	0.04	1.8	0.02
MAPK signaling pathway	39	492	173	2862	2.2	0.06	1.3	0.04
Toll-like receptor signaling pathway	16	492	58	2862	0.9	0.06	1.6	0.03
Colorectal cancer	17	492	63	2862	1	0.06	1.6	0.03
ECM-receptor interaction	13	492	44	2862	0.7	0.06	1.7	0.03
Natural killer cell mediated cytotoxicity	18	492	68	2862	1	0.06	1.5	0.03
Fc gamma R-mediated phagocytosis	17	492	64	2862	1	0.07	1.5	0.04
Arachidonic acid metabolism	10	492	31	2862	0.6	0.07	1.9	0.03
VEGF signaling pathway	14	492	50	2862	0.8	0.07	1.6	0.04

Table 7

Gene ontology term	Count	LT	PH	PT	%	P-Value	Fisher Exact	Fold Enrichment
Defense response	50	905	133	4828	2.8	4.80E-07	1.80E-07	2
Inflammatory response	31	905	71	4828	1.7	3.40E-06	1.00E-06	2.3
Innate immune response	20	905	37	4828	1.1	7.20E-06	1.50E-06	2.9
Regulation of RNA metabolic process	116	905	432	4828	6.5	1.50E-05	9.10E-06	1.4
Regulation of transcription	157	905	623	4828	8.8	1.70E-05	1.10E-05	1.3
Regulation of transcription, DNA-dependent	112	905	421	4828	6.3	3.30E-05	2.10E-05	1.4
Response to wounding	42	905	120	4828	2.3	3.50E-05	1.50E-05	1.9
Complement activation	9	905	14	4828	0.5	1.50E-03	2.20E-04	3.4
Activation of plasma proteins involved in acute inflammatory response	9	905	14	4828	0.5	1.50E-03	2.20E-04	3.4
Transcription	82	905	323	4828	4.6	2.10E-03	1.40E-03	1.4
Protein maturation by peptide bond cleavage	13	905	28	4828	0.7	2.70E-03	7.40E-04	2.5
Activation of immune response	15	905	35	4828	0.8	2.70E-03	8.40E-04	2.3
Immune response	47	905	169	4828	2.6	3.80E-03	2.20E-03	1.5
Humoral immune response mediated by circulating immunoglobulin	7	905	10	4828	0.4	4.50E-03	5.60E-04	3.7
Complement activation, classical pathway	7	905	10	4828	0.4	4.50E-03	5.60E-04	3.7
Humoral immune response	9	905	16	4828	0.5	4.50E-03	8.90E-04	3
Acute inflammatory response	14	905	33	4828	0.8	4.50E-03	1.40E-03	2.3
Modification-dependent protein catabolic process	39	905	136	4828	2.2	5.00E-03	2.80E-03	1.5
Modification-dependent macromolecule catabolic process	39	905	136	4828	2.2	5.00E-03	2.80E-03	1.5
Adaptive immune response	13	905	30	4828	0.7	5.40E-03	1.60E-03	2.3
Adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	13	905	30	4828	0.7	5.40E-03	1.60E-03	2.3
Cellular macromolecule catabolic process	51	905	191	4828	2.9	6.00E-03	3.70E-03	1.4
Immunoglobulin mediated immune response	11	905	24	4828	0.6	7.80E-03	2.20E-03	2.4
Leukocyte mediated immunity	13	905	32	4828	0.7	9.80E-03	3.30E-03	2.2
B cell mediated immunity	11	905	25	4828	0.6	0.01	3.20E-03	2.3
Respiratory gaseous exchange	5	905	6	4828	0.3	0.01	1.20E-03	4.4
Protein modification by small protein conjugation or removal	15	905	41	4828	0.8	0.01	5.40E-03	2
Protein processing	15	905	41	4828	0.8	0.01	5.40E-03	2
Proteolysis involved in cellular protein catabolic process	41	905	154	4828	2.3	0.02	9.20E-03	1.4
Lymphocyte mediated immunity	12	905	30	4828	0.7	0.02	5.40E-03	2.1